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14. ABSTRACT The hypothesis of this study is that glucosylceramide synthase (GCS)-governed drug resistance can be acquired through exposure to ceramide-generating anticancer agents(e.g., Adriamycin, paclitaxel, and etoposide). We have shown that paclitaxel, Adriamycin, and SDZ PSC 833 induce ceramide generation in MCF-7 cells (200, 83, and 900%, respectively, at 24h). Paclitaxel and SDZ PSC 833 increased ceramide levels by 192 and 460%, respectively, at 24h in MDA-MB-231 cells. The ceramide generated by Paclitaxel and SDZ PSC 833 is metabolized to glucosylceramide (GC) (54 and 200%, respectively, in MCF7 cells at 24h; 172 and 307%, respectively, at 24h in MDA-MB-231 cells). When wild-type breast cancer cells such as MCF-7 and MDA-MB-231 are exposed to a low concentration of [14C]C6-ceramide (0.2 g/ml), cells converted it to sphingomyelin. However, when breast cancer cells are challenged with a higher concentration of [14C]C6-ceramide (5 g/ml), ceramide was glucosylated to GC via GCS. Acute exposure of MCF-7 and MDA-MB-231 cells to C8-GC (10 g/ml) for 72h increased MDR1 expression by 2- and 4-fold, respectively. Chronic exposure of MDA-MB-231 cells to C8-ceramide for extended periods induced a dramatic increase in MDR1 mRNA levels, and also elicited expression of P-glycoprotein. High passage C8-ceramide-grown MDA-MB-231 cells were more resistant to Adriamycin and paclitaxel. These experiments show that high levels of ceramide enhance expression of the MDR phenotype in cancer cells through what we propose is a GC intermediate. We then studied another major avenue of ceramide elimination, hydrolysis via ceramidase (CDase). In MCF-7/VP (etoposide resistant) and MCF-7/CDDP (cisplatin resistant) compared to MCF-7 cells, alkaline CDase increased by 2- and 3-fold, respectively, and in MCF-7/Adr cells, neutral CDase increased by 3.4-fold. This result suggests that CDase could be implicated also in the drug-resistant phenotype.					
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INTRODUCTION

Poor response to chemotherapy is a major clinical problem, and in most instances drug resistance is the underlying cause [1]. This is a most undesirable situation, and patients and oncologists would welcome its possible correction. In breast cancer nearly 50% of patients demonstrate primary and/or secondary resistance to Adriamycin (doxorubicin) [2]. Several mechanisms of drug resistance are being examined, and avenues to reverse resistance are being sought. Research strategies in this area have become increasingly aimed at molecular targets such as P-glycoprotein (P-gp), multidrug resistance associated protein (MRP), topoisomerase, and Bcl-2 protooncogene, to name a few. Overexpression of the membrane efflux transporter, P-gp, is one of the most consistent biological alterations in drug resistance [1,3]. P-gp (170 kDa), the product of the *MDR1* gene, is an energy-dependent pump that reduces the intracellular concentration of specific anticancer drugs, and it has been studied extensively. Our approach to drug resistance involves ceramide metabolism. Ceramide, a neutral lipid, has been shown to play a role in signaling apoptosis [4]. Ceramide can be generated by two different pathways. The first one is the *de novo* pathway, whereby serine and palmitoyl-CoA condense through a series of reactions, generate dihydroceramide and ceramide, respectively. The second one is the salvage pathway, which involves the hydrolysis of membrane sphingomyelin (SM) by sphingomyelinase (SMase). Once ceramide is generated, it can be converted into a number of other bioactive sphingolipids, including ceramide-1-phosphate (by ceramide phosphorylation via ceramide kinase), glucosylceramide (by ceramide glycosylation via glucosylceramide synthase), and sphingosine (by ceramide hydrolysis via ceramidase). Several front-line anticancer agents elicit the formation of ceramide [4,5]. If ceramide formation in response to drug treatment is blocked, then the cytotoxic impact of the drug is largely reduced [6,7]. This demonstrates ceramide's role in drug responses. Ceramide added directly to cells circumvents the enzyme route of ceramide formation and promotes an apoptotic cascade directly [7,8]. Our group showed that increased cellular capacity for ceramide glycosylation, catalyzed by glucosylceramide synthase (GCS), is associated with chemotherapy resistance in cancer cells [9-13]. In cultured breast cancer cells, sensitivity to anthracyclines and taxanes can be decreased or increased simply by manipulation of GCS activity [11,14,15]. For example, transfection of drug sensitive MCF-7 breast cancer cells with GCS cDNA confers resistance to Adriamycin [14], and transfection of multidrug resistance MCF-7-AdrR breast cancer cells with antisense GCS (asGCS) increases cell sensitivity to chemotherapy by a factor of 28-fold for Adriamycin, more than 100-fold for vinblastine, and more than 200-fold for paclitaxel [11].

BODY

Deviation from the original statement of work

Due to some problems during the realization of my proposal, I was not being able to accomplish certain elements.

1. Problems encountered in the assaying of acidic ceramidase necessitated that I modify the *in vitro* enzyme conditions (the neutral and the alkaline enzymes have been successfully evaluated).
2. Excessive down-time with the liquid scintillation counter LS 330 that we use to trace tritium and carbon 14-labeled cellular ceramide, sphingosine, and glycolipid metabolites, an important aspect of my proposal. New equipment is on order.

I requested a 6-month no-cost extension, and all the data will be reported in the Final Report to be submitted January 2008.

Key Research Accomplishments

1- Determination of whether ceramide and glucosylceramide generation are influenced by paclitaxel, cisplatin, Adriamycin, etoposide, or SDZ PSC 833 in breast cancer cells.

MCF-7 cells were treated separately with Adriamycin (2.5 M), cisplatin (CDDP) (10 M), etoposide (VP16) (1.0 M), paclitaxel (1.5 M), and SDZ PCS 833 (5.0 M) for 30min, 4 and 24h in medium containing [³H] palmitate as metabolic tracer. In MCF-7 cells, CDDP and VP16 did not induce ceramide generation at any treatment time. We observed that paclitaxel induced a significant 2-fold increase in ceramide generation after 24h. Doxorubicin generated an 83% elevation in ceramide at 24h. With SDZ PSC 833 ceramide production increased by 2-, 3.9-, and 9-fold at 30min, 4, and 24h, respectively (Fig. 1A), illustrating a time-dependent response. Only drugs that elicit a significant increase in ceramide induced GC production. GC production increased 54% after 24h treatment with paclitaxel, and SDZ PCS 833 induced a 2-fold increase at 4, and 24h (Fig. 1B). Even though doxorubicin generated ceramide at 24h in MCF-7 cells, GC production was not observed.

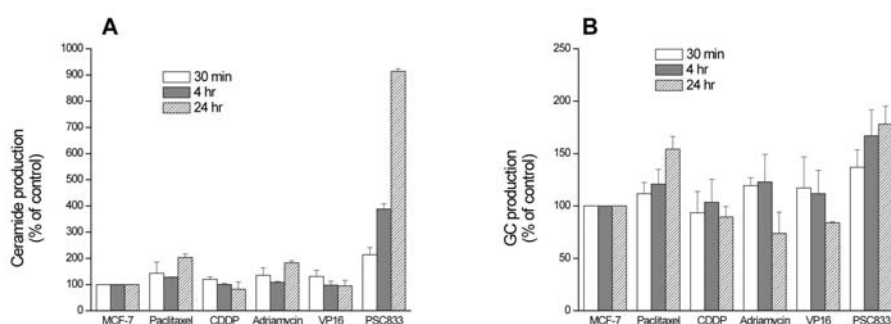


Fig. 1. Influence of anticancer drugs on ceramide and glucosylceramide production. MCF-7 cells were seeded in 6 well-plates (750,000 cells/well) with or without paclitaxel (1.5 μ M), CDDP (10 μ M), Adriamycin (2.5 μ M), VP16 (1.0 μ M), and SDZ PSC 833 (5.0 μ M) in culture medium containing 2.0 μ Ci/ml [³H]palmitic acid for the indicated times. Ceramide (A) and GC (B) were resolved from total lipid extracts by TLC.

To determine whether the induction of ceramide by chemotherapy drugs in breast cancer cells was cell-type dependent, we assessed ceramide and GC production in another breast cancer cell line, MDA-MB-231 (ER(-) cell line). MDA-MB-231 cells demonstrated similar responses compared to MCF-7 cells regarding ceramide and GC production via paclitaxel, cisplatin, Adriamycin, etoposide, and SDZ PSC 833 exposure (Fig. 2). At 30min, only SDZ PSC 833 increased ceramide generation (+72%) compared to untreated cells. SDZ PSC 833 continued to promote time-dependent increases in ceramide 2.6- and 4.6-fold at 4 and 24h, respectively (Fig. 2A). MDA-MB-231 cells responded to SDZ PSC 833 with significant increases in GC levels at 4h (+267%) and 24h (+307%) (Fig. 2B). Increases in ceramide (+192%) and GC (+171%) were also found in MDA-MB-231 cells treated with paclitaxel (24h), and Adriamycin induced a 2.3-fold increase in ceramide production at 24h without any elevation in GC level.

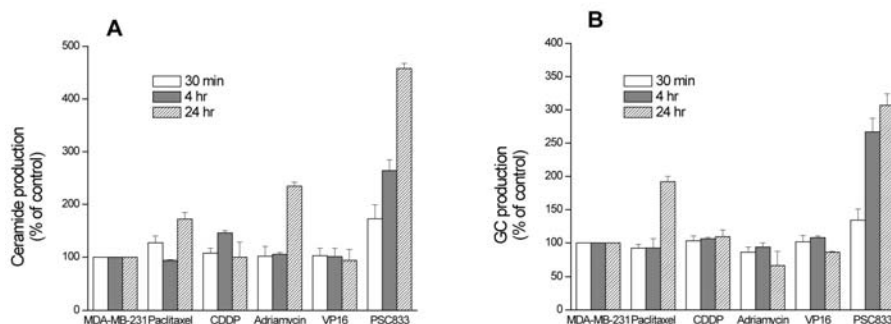


Fig. 2. Influence of anticancer drugs on ceramide and glucosylceramide production. MDA-MB-231 cells were seeded in 6 well-plates (500,000 cells/well) with or without paclitaxel (1.5 μ M), CDDP (10 μ M), Adriamycin (2.5 μ M), VP16 (1.0 μ M), and SDZ PSC 833 (5.0 μ M) in culture medium containing 2.0 μ Ci/ml [³H]palmitic acid for the indicated times. Ceramide (A) and GC (B) were resolved from total lipid extracts by TLC.

2- Cellular metabolism of short-chain ceramide.

To obtain a more detailed analysis of ceramide metabolism in breast cancer cells, including possible conversion to sphingomyelin (SPM) and lactosylceramide (LacCer), we exposed MCF-7 and MDA-MB-231 cells to [14 C]C6-cer. The [14 C]C6-cer was diluted with unlabeled C6-cer to obtain a concentration in the medium of 5.0 g/ml. In Figure 3, MDA-MB-231 cells converted 80% of the counts taken up to [14 C]C6-GC after 24h, and MCF-7 cells show a conversion rate of 62%. Both cell lines synthesized [14 C]C6-LacCer up to 10% of the total counts. MCF-7 and MDA-MB-231 cells synthesized C6-SPM from [14 C]C6-cer, with counts ranging from 8 to 18% of the total radioactivity.

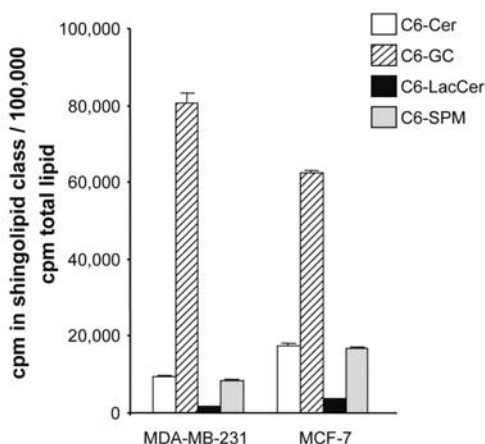


Fig. 3. Metabolism of [14 C]C6-ceramide in MCF-7 and MDA-MB-231 cells. Cells in 6-well plates were grown with [14 C]C6-ceramide (500,000 cpm/ml, 5 μ g/ml) for 24 h, after which total lipids were extracted and analyzed by TLC for radiolabeled free C6-ceramide, C6-GC, C6-SPM and C6-LacCer.

3- Influence of C8-ceramide (C8-cer) and glucosyl C8-ceramide (C8-GC) on MDR1 expression in MCF-7 and MDA-MB-231 cells.

To determine whether ceramide and GC could influence MDR1 expression, we treated MCF-7 and MDA-MB-231 cells with short-chain analogs C8-cer and C8-GC. Acute exposure to C8-cer did not alter MDR1 expression in MCF-7 cells, whereas exposure of MDA-MB-231 cells to C8-cer (5.0 g/ml) was cytotoxic (data not shown). We then investigated whether GC, a known P-gp substrate, would regulate MDR1 expression. Acute exposure of MCF-7 and MDA-MB-231 cells to C8-GC (72h) resulted in 2- and 4-fold increases, respectively, in MDR1 mRNA levels (Fig. 4).

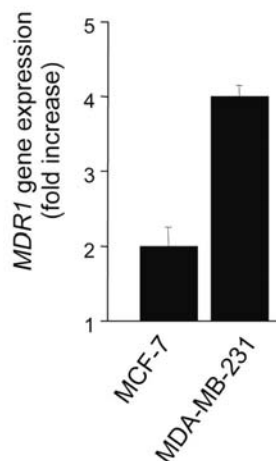


Fig. 4. Influence of C8-GC on MDR1 expression in MCF-7 and MDA-MB-231 cells. Cells (500,000) were seeded into 6-cm dishes and the following day supplemented with C8-GC (10 μ g/ml medium). After 72 h, total RNA was extracted and analyzed by qPCR. Fold increases in MDR1 were calculated using actual number of gene copies per unit β -actin expression.

4- Ceramide influence on MDR1 phenotype.

The data of Figure 4 demonstrate the high capacity of MDA-MB-231 cells to generate [14 C]GC from [14 C]C6-cer supplements. We used this model to assess whether ceramide via conversion to GC would influence expression of MDR1. MDA-MB-231 cells were first grown with 2.5 g/ml doses of C8-cer for two passages, after which the dose was increased to 5.0 g/ml, with minimal cytotoxicity. Prolonged growth of MDA-MB-231 cells in C8-ceramide-containing medium caused a robust increase in MDR1 mRNA levels. Figure 5A demonstrates, by gel electrophoresis of RT-PCR products, the dramatic increase in the level of MDR1 expression in MDA-MB-231/C8cer cells (at passage 22) compared to wild-type MDA-MB-231 cells. In order to determine if upregulation of MDR1 mRNA levels resulted in enhanced expression of P-gp protein, Western blot analyses were conducted. The results of Figure 5B show that under the conditions employed, P-gp was undetectable in wild-type MDA-MB-231 cells; however, P-gp levels increased with increased C8-ceramide exposure time (passages 12 and 22). These data show that C8-ceramide exposure upregulates both MDR1 mRNA and protein.

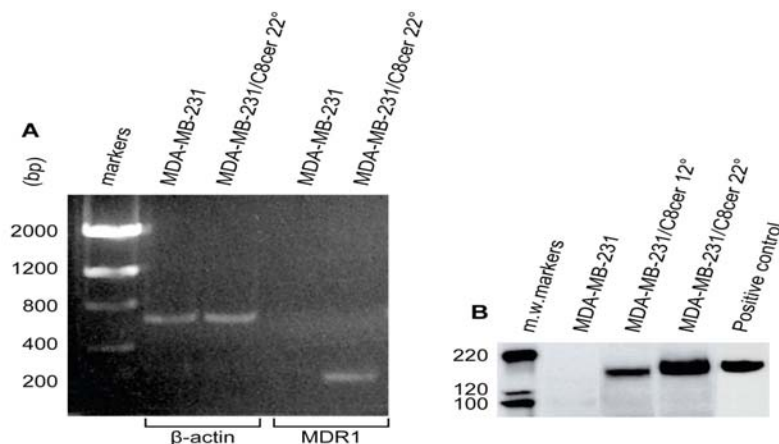


Fig. 5. The influence of chronic exposure to C8-ceramide on MDR1 mRNA, P-gp levels in MDA-MB-231 cells. (A) MDR1 mRNA levels by RT-PCR in MDA-MB-231 cells and in high passage C8-ceramide cells (MDA-MB-231/C8cer, passage 22). Samples were subjected to RT-PCR analysis (0.5 µg RNA/tube) and products were resolved on 1% agarose gels. β-actin was employed as housekeeping gene. (B) P-gp levels by Western blot in MDA-MB-231 and in MDA-MB-231/C8cer cells at passages 12 and 22. Aliquots (100 µg cell protein) were electrophoresed for Western blot analysis of P-gp (C219 antibody). KB-ChR-8-5 (colchicine-resistant human epidermoid carcinoma) cell protein (50 µg) was used as a positive control for P-gp.

5- Resistance to doxorubicin and paclitaxel in MDA-MB-231/c8cer cells compared to MDA-MB-231 cells.

Chemotherapy sensitivity status of MDA-MB-231/C8cer cells was evaluated and compared with wild-type MDA-MB-231 cells. Doxorubicin and paclitaxel, natural product chemotherapy drugs that are substrates for P-gp, were used. MDA-MB-231/C8cer cells were more resistant to anticancer drugs. As shown by dose-response/cell viability curves (Fig. 6), MDA-MB-231/C8cer cells were approximately 3- and 9-fold more resistant to doxorubicin and paclitaxel, respectively, compared to MDA-MB-231 cells. Values of IC₅₀ were 88 and 270 nM for doxorubicin in MDA-MB-231 and MDA-MB-231/C8cer cells, respectively, and 36 and 331 nM for paclitaxel in MDA-MB-231 and MDA-MB-231/C8cer cells, respectively.

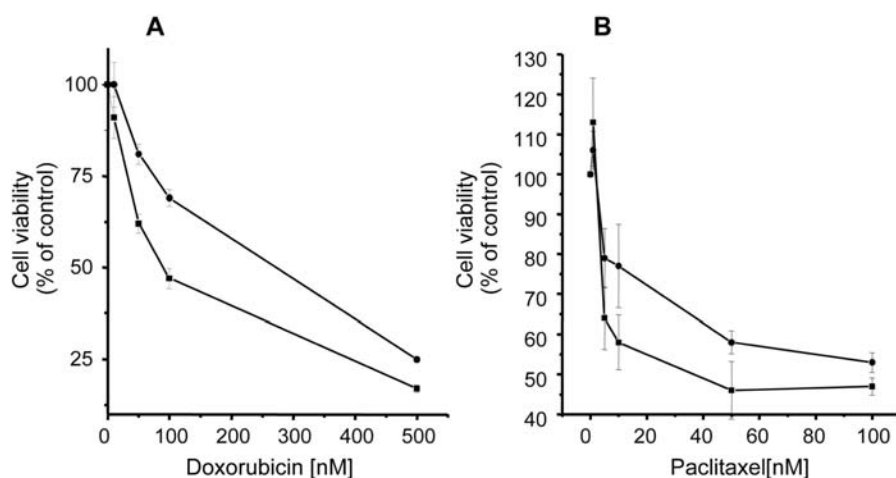


Fig. 6. Doxorubicin and paclitaxel sensitivity in MDA-MB-231 (■) and MDA-MB-231/C8cer cells (●). MDA-MB-231/C8cer cells (passage 17) were used in the chemosensitivity assays, (A) doxorubicin and (B) paclitaxel. C8-ceramide was not in the medium during the experiment. Cell viability was determined by MTS assay.

6- Metabolism of [14 C]C6-ceramide by drug resistant breast cancer cells.

We exposed the various drug resistant cell lines to [14 C]C6-cer at a low concentration (0.2 g/ml). MCF-7, MCF-7/VP, MCF-7/CDDP, and MCF-7/AdrR cells took up 14.3, 14.2, 19, and 10.2% of [14 C]C6-cer, respectively, and converted 49, 76, 95, and 37% of that to short-chain sphingomyelin (Fig. 7). Therefore, when cells are not challenged with excessive amounts of ceramide (as in the 5 μ g/ml regimen), the conversion to short-chain GC is very low.

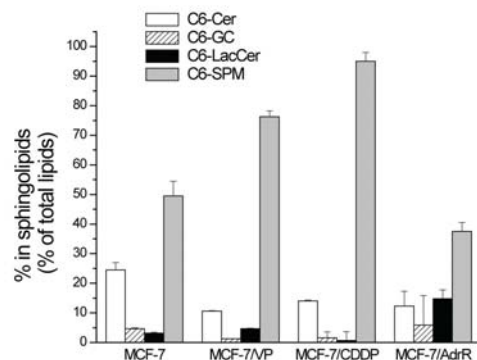


Fig. 7. Metabolism of [14 C]C6-ceramide in MCF-7 and drug resistant MCF-7 cells. Cells in 6-well plates were grown with [14 C]C6-ceramide (84,500 cpm/well, 0.2 μ g/ml) for 24 h, after which total lipids were extracted and analyzed by TLC for radiolabeled free C6-Cer, C6-GC, C6-LacCer, and C6-SPM.

7- Determination of ceramidase expression in MCF-7 and drug resistant MCF-7 cells.

Another major avenue of ceramide elimination is by hydrolysis via ceramidase (CDase). Ceramidases hydrolyze ceramide into sphingosine, a precursor of the antiapoptotic factor sphingosine-1-phosphate, and free fatty acid. The acid ceramidase is located within lysosomes, neutral ceramidase is localized mainly in plasma membrane, and the alkaline ceramidase is in the Golgi apparatus.

In Figure 8, we assessed ceramidase expression in MCF-7 cells and in drug resistant MCF-7 variants (by real-time RT-PCR). MCF-7/VP showed a 2-fold increase in alkaline CDase expression compared to MCF-7 cells. A 3-fold increase was shown for MCF-7/CDDP cells compared to their wild-type MCF-7/P. In MCF-7/AdrR compared to MCF-7 cells, levels of expression of alkaline CDase did not change, whereas acid CDase expression decreased by 2.6-fold. The major difference between MCF-7/AdrR and MCF-7 was the neutral CDase, which was 3.4-fold higher in drug resistant cells.

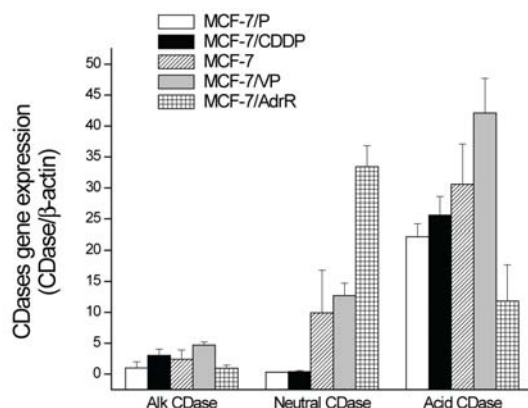


Fig. 8. Expression of ceramidase isoforms in MCF-7 and drug resistant MCF-7 cells. Total RNA from MCF-7/P, MCF-7/CDDP, MCF-7, MCF-7/VP, and MCF-7/AdrR cells was extracted and analyzed by real-time RT-PCR.

Key Research Accomplishments

Determined whether ceramide and glucosylceramide metabolism (generation) is influenced by paclitaxel, cisplatin, Adriamycin, etoposide, or SDZ PSC 833 (a P-glycoprotein antagonist) in breast cancer cells.

Determined the cellular metabolic route of ceramide in breast cancer cells (using a short-chain analog).

Determined the influence of C8-cer and C8-GC on MDR1 expression in MCF-7 and MDA-MB-231 cells (ER(+), ER(-), respectively).

Determined the influence of ceramide on MDR1 phenotype in breast cancer cells.

Determined the cytotoxic responses to doxorubicin and paclitaxel in MDA-MB-231/c8cer cells compared to MDA-MB-231 cells, the former being made P-gp-expressing as a result of chronic exposure to C6-ceramide.

Determined the metabolic route of [14 C]C6-ceramide in drug resistant breast cancer cells (MCF-7-AdrR).

Determined ceramidase expression (three isoforms) in MCF-7 and drug resistant MCF-7 counterparts.

Reportable Outcome

1. "Sphingolipids can influence breast cancer cell phenotype" (poster presentation). 4th International Charleston Ceramide Conference. Pacific Grove, California, March 7-11, 2007.

Conclusion

We have shown that some anticancer drugs promote the generation of ceramide in breast cancer cells (MCF-7 and MDA-MB-231). When MCF-7 and MDA-MB-231 cells are exposed to a low level of ceramide, the ceramide is metabolized into sphingomyelin. When the cells are challenged with a high level of ceramide, it is metabolized to glucosylceramide. This demonstrates the versatility of breast cancer cells for shunting ceramide into multiple metabolic pathways. Our work also shows that high levels of ceramide enhance expression of the multidrug resistance phenotype in breast cancer cells through the generation of GC. Therefore, ceramide's role as a messenger of cytotoxic response might be linked to the multidrug resistance pathway.

Another pathway of ceramide elimination is by hydrolysis. We have demonstrated differences in gene expression of ceramidases between drug resistant and wild-type breast cancer cells, but the determination of the enzymatic activity in vitro, using cell-free systems will more clearly emphasize the importance of this pathway in the drug-resistant breast cancer cells.

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